IDENTIFICATION OF CIRCULATORY MICRORNAs AS NOVEL BIOMARKERS IN SOUTH INDIAN WOMEN WITH PCOS

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Identification of circulatory micro RNAs as novel bio-markers in South Indian women with PCOS

Introduction :

Polycystic ovarian syndrome (PCOS) is the most common endocrine and metabolic heterogeneous disorder in women of reproductive age, with a likely genetic origin influenced by environmental factors such as diet, lifestyle habits, and socio-economic status[1]. The complications of PCOS include abnormal uterine bleeding, infertility, endometrial cancer and late menopause. In addition PCOS patients often have mood changes and emotional disturbance, low self-esteem, anxiety, and metabolic disorders like obesity, impaired glucose tolerance, type 2 diabetes, dyslipidemia and high blood pressure [2]. The prevalence of this disorder varies from 6% to 15% depending on the diagnostic criteria used. The prevalence is as high as 15% when the broader Rotterdam criteria are applied[3].

Rotterdam criteria

The European Society of Human Reproduction and Embryology/ American Society of Reproductive Medicine held a consensus meeting at Rotterdam in 2003 and developed revised criteria incorporating a wider spectrum of phenotypes in PCOS[4]. Two out of three criteria would be needed to make the diagnosis after exclusion of the other causes of hyperandrogenism.

Oligoanovulation and /or anovulation.

Clinical and /or biochemical signs of hyperandrogenism.

Polycystic ovarian morphology by ultrasound.

Exclusion of other etiologies of androgen excess and anovulatory infertility is necessary

Ultrasonography Assessment of Polycystic Ovarian Morphology[4].

The 2003 Rotterdam criteria for polycystic Ovarian morphology recommends the presence of 12 or more follicles in each ovary measuring 2 to 9 mm in diameter and /or increased ovarian volume(›10 ml). The Transabdominal route can be used to measure ovarian volume.

Biochemical sign of hyperandrogenism

Serum total Testosterone provides the best estimate of the androgen status. Other measurements to assess androgen excess in women with PCOS includes free androgen index (FAI). FAI is defined as the ratio between total testosterone and Sex Hormone Binding Globulin (SHBG) and can also be used to evaluate hyperandrogenemia. Low SHBG level reflects insulin resistance and predicts the susceptibility for developing metabolic syndrome[4].Low SHBG also increases free

testosterone levels in target tissues. In the past increased LH/FSH (Luteinising Hormone/Follicle Stimulating Hormone) ratio has been used for diagnosis of PCOS. However LH secretion is pulsatile and a single measurement of LH can miss the intrinsic variability. Hence an increased LH/FSH ratio contributes to the diagnosis of PCOS, but the absence does not rule out the diagnosis of PCOS.

Circulatory miRNA Expression

MicroRNAs (miRNAs) are 22-nucleotide endogenous noncoding RNAs that create faulty base pairings with selected regions on target mRNAs' 3' UTRs. MicroRNAs may serve as biomarkers to differentiate PCOS patients from healthy menstrual women or to pinpoint sub-phenotypes within this complex condition[5]. These epigenetic regulators play a key role in regulating ovarian follicle size, atresia, ovulation, and steroidogenesis in the female reproductive system. MiRNAs such as miR-132, miR-320, miR-520c-3p and miR-483-5p were shown to be differentially regulated in PCOS patients when hormone balance was altered[5]. Exosomes, which are thought to be mediators for intercellular communication, secrete these miRNAs into the circulatory system. ExomiRs are expressed differently in obese and lean PCOS women when compared to healthy women. PCOS has a multifaceted effect, and exosomes are implicated in various clinical processes, such as chronic inflammation and insulin resistance[6]. Alterations in the micro RNAs that are circulating in the blood reflect significant changes in the tissues that are located in the periphery, and these alterations are being considered as potential biomarkers in the same way that enzymes and cytokines are in obesity-related metabolic diseases[5]. Significant difference in the levels of miRNAs in anovulatory PCOS women was reported when compared to their age and BMI matched control counterparts[5]. Even though many reports have postulated the changes in small noncoding RNAs (miR) in the etiopathogenesis of polycystic ovarian syndrome (PCOS), the findings were contradictory[5]. As a result, the purpose of the current study was to determine whether or not miR levels differed in serum samples collected from women with PCOS and controls who were of the same age.

Aim :

To study microRNA dysregulation networks to find epigenetic predisposition in reproductive age group PCOS women.

Objectives :

To evaluate the free androgen index and its role in PCOS correlative with miRNA expression.
To study the metabolic and reproductive complications of PCOS in south Indian women

Hypothesis:

we hypothesize that the expression of circulatory microRNAs varies in women with PCOS when

compared to women without PCOS.

Research Question:

Can differential expression of circulatory miRNAs be used as a biomarker in the diagnosis of women with predisposition for PCOS?

Review of literature :

The etiology of polycystic ovarian syndrome is uncertain .There is some evidence of autosomal transmission related to strong family clustering. Potentially a gene or series of genes renders the ovaries susceptible to insulin stimulation of androgen secretion while blocking follicular maturation. Women genetically predisposed to polycystic ovarian syndrome fail to resume normal insulin sensitivity and continue to express metabolic and endocrine features usually confined to puberty. Recent studies indicate that this disorder could be a complex trait. This means that several genes are interacting with environmental factors to provoke the gentype[7]. In contrast, biochemical parameters, including fasting insulin levels or hyperandrogenemia, seem to be highly inheritable parameters, suggesting that some clinical signs, symptoms, or biochemical parameters of PCOS could be transmitted as Mendelian autosomal dominant or X-linked traits, but the genetic studies have not as yet concluded the pattern of heredity[8]. While studies, so far, are unable to exclude an autosomal or X-linked dominant mode of inheritance, the heritability of PCOS is probably more complex, similar to that of type 2 diabetes mellitus or cardiovascular disease. However, a positive family history appears to be the most informative risk factor for the development of PCOS. Furthermore, environmental factors alter the clinical and biochemical presentation in those with genetic predisposition to PCOS. There are different candidate genes as a cause of PCOS: such as genes involved in steroid hormone synthesis and action, genes involved in carbohydrate metabolism and fuel homeostasis, genes involved in gonadotropin action and regulation and genes in the major histocompatibility region, which could account for certain PCOS features. Different studies have indicated genetic susceptibility to PCOS. It was shown that polycystic ovaries and hyperandrogenemia are present in 50% of sisters of affected women. Therefore genetic analyses of candidate genes have been performed. Both linkage and association studies have suggested that PCOS can be explained by the interaction of a small number of key genes with environmental, particularly nutritional factors. Hyperandrogenemia is genetically determined and the result of familial studies indicate that hyperandrogenism clusters as a dominant genetic trait[9].Finally abnormalities in gonadotropin secretion, particularly LH, are

characteristic of PCOS, which promotes exploring genes related to the regulation of LH secretion, LH bioactivity, and LH action.

MicroRNAs (miRNA) are endogenous, small, non-coding, single-stranded, regulatory RNA molecules, composed of 20-24 nucleotides. Processed from larger stem-loop precursor transcripts, miRNAs modulate gene expression post-transcriptionally by binding to the 3' untranslated region of target mRNA, thus inhibiting translation, inducing mRNA destabilization or both[10]. Recently miRNAs have been highlighted in the field of disease biomarkers. Extracellular miRNAs circulate freely in the blood, and measurements of circulating miRNAs have the advantage of stability and easy detection in plasma or serum[11]. Therefore, blood miRNA may serve as a minimally invasive biomarker for the diagnosis of various diseases and lead to a greater understanding of the molecular mechanisms underlying the pathogenesis of diseases. Several studies on the altered expression of miRNAs have been reported in women with PCOS. The expression of whole blood miRNA-21, miRNA-27b, miRNA-103, and miRNA-155 was significantly increased in obese women with PCOS compared with lean women with PCOS. Serum free testosterone levels were positively associated with the expression of whole blood miRNA-21, miRNA-103 and miRNA-155[12]. Although serum miRNA-222, miRNA-146a and miRNA-30c were suggested as novel biomarkers for the diagnosis of PCOS, few studies have been conducted on the altered expression of serum miRNAs in women with PCOS[13]. Circulating miRNAs are very stable to enzymatic degradation and physical conditions such as Freeze/Thaw and can be stored for months without their degradation[14]. As such they are attractive as biomarkers for disease diagnosis and monitoring. Therefore specific miRNA or combination of miRNAs may become a reliable marker for the diagnosis of PCOS, which may help distinguish the different phenotypes identified by the Rotterdam criteria[15]. miRNA may also be used as a biomarker to support positive diagnosis of PCOS rather than the diagnosis being one of exclusion that is currently the case, which would be of value in adolescence or in the perimenopausal/menopausal women where diagnosis is difficult[15].

Research gaps/Lacunae:

There are lot of Chinese and western literature available on study of miRNA dysregulation in PCOS but so far not many Indian studies are available on this[1-15]. These miRNA may have a possible role in polycystic ovarian syndrome, insulin resistance and infertility[15]. A greater understanding of the underlying molecular mechanism may help to improve diagnosis and

treatment of this syndrome. Present abundantly in serum, miRNAs could serve as a noninvasive marker for PCOS, as they have been shown to be stable in serum, resistant to nuclease activity and are easy to detect[15]. MicroRNAs could be used as bio-markers distinguishing PCOS patients from normal menstruating women or to identify sub-phenotypes within this heterogeneous syndrome. Furthermore, insight into the interplay between epigenetic regulation and miRNAs leading to an altered miRNA expression could perhaps help to explain the pathogenesis behind the disease.[15] The dynamic expression of miRNAs has been shown to be influenced by several factors such as LH/hCG, FSH, androgens and cAMP *in vitro*. In addition, identifying upstream or additional regulators of miRNAs, their target genes and their roles in the related signaling pathways will further shed light on the importance of specific miRNAs for both the development and function of the ovary, paving the way for new therapeutic strategies by controlling the key factors in the regulatory networks. Furthermore recent studies have demonstrated that SNPs located in miRNA genes or miRNA binding sites potentially modify miRNA regulation thus affecting phenotypes and disease susceptibility. Therefore a combination of miRNA expression profiles with genome wide SNP genotyping might help distinguish among potential disease related biomarkers. A better understanding of the regulation of ovarian function by miRNAs may offer a theoretical foundation for ovarian diseases.

Methodology :

This study was planned as a case-control study.

Institute ethical committee and IRB approval was obtained.

Place of study-Shri Satya Sai Medical College and Research institute.

Study Population-women in the age group 15-45 years with complaints s/o PCOS and age matched control group

Source of data-women in the age group 15-45 years attending gynec OPD at SSSMCRI.

Sample size calculated to be 50 cases of PCOD plus 50 cases of age matched controls in the age group 15-45 years.

Sample size calculation

$$n = \frac{(Z_{1-\alpha 2})^2 \, P(1-P)}{d^2}$$

P-Prevalence-15%-0.15[16]

α-Type 1 error-0.05- Z1-α2 -1.96

Precision d-10%

$N = \frac{1.96^2 \; x \; 0.15 \; x \; 0.85}{0.1^2} = 49$

Sample size-50

Inclusion criteria-

Women in the age group 15-45 years. PCOS cases were diagnosed using Rotterdam criteria and the controls were age matched women without PCOS.

All of the control women had regular periods, no clinical or biochemical hyperandrogenemia, no significant background history and none of them were on any medications including oral contraceptive pills.

Exclusion criteria-

Women who are already on treatment for PCOS or on oral contraceptive pills /Hormonal treatment.

Informed written consent was taken from all the participants in the study. All the women were subjected to a detailed history taking including age, detailed menstrual history, Obstetric history if any, family history etc. These women were subjected to a physical examination including height, weight , BMI, waist, hip circumference, signs of hirsutism (based on modified Ferriman-Gallwey score-significant hirsutism-Total score ≥8), acne was scored based on its number, type and distribution, premature alopecia, acanthosis nigricans and blood pressure. Systemic and gynaecological examination was done .The women were further subjected to an ultrasound examination of the ovaries (transabdominal-if necessary transvaginal) on the second or third day of periods. Routine basic investigations were done. Venous blood samples were obtained after an overnight fast from each participant on the second or third day of her spontaneous or progesterone induced menstrual period. Hormonal assay was done including Total Testosterone (TT) and Sex hormone binding globulin(SHBG) .FAI was calculated using the formula: TT (nmol/lt) X 100/SHBG (nmol/lt). The sera was stored at -20^0c and later transported to lab where it was stored at -80^0c. The microRNA was estimated using a two stage process.

Differential expression of miRNA in serum :(To identify expression of different miR in PCOS and control to select differentially expressed miR)

Microarray: Total RNA was extracted from serum using TRIzol reagent. The concentration of the RNA was analyzed by NanoDrop and the RNA integrity was checked on Bioanalyzer 2100 (Agilent, USA). miRNAs was selected based on more than 1.5-fold upregulation or less than

0.67-fold downregulation in women with PCOS compared with controls.

<u>Expression of- selected circulatory miR in serum</u> :(To evaluate expression of selected miR in PCOS and control for use as biomarker)

Quantitative real-time PCR: Total RNA was isolated from serum using TRIzol reagent. Small RNAs were separated using enrichment columns. The concentration of the RNA was measured using NanoDrop and the RNA integrity was checked on Bioanalyzer 2100 (Agilent, USA).

miRNA microarrays:

The samples were processed using TRIzol reagent (manufactured by Invitrogen) in order to extract total RNA. The total amount of RNA extracted from each sample that was used for the follow-up microarray was two hundred nanograms (200 ng). Poly (A) polymerase, also known as PAP, was applied to the 3' end of each sequence of total RNA in order to attach a length of Poly-A tail. In order to produce biotinylated cDNA, an Ambion Illumina TotalPrep RNA Amplification Kit was applied to the reaction. Primers for 1146 different human miRNAs were included in the microRNA expression profiling kit .cDNAs that had been biotinylated were hybridised with oligonucleotides that were specific to microRNA. The oligos that were not bound were removed by washing, followed by extension and ligation reaction. After performing Polymerase Chain Reactions (PCR) with fluorescently labelled universal primers, the fluorescently tagged, single-stranded PCR products were hybridised to capture beads. This was followed by the collection of the DNA. After that, the iScan System from Illumina was used to detect the fluorescent signals.

1-Multi-Class Dif (RVM-F test):

The raw data from each array was evaluated by employing a technique called Multi-Class Dif, also known as the RVM-F test, which is suited to the analysis of small sample sizes for several groups. In order to identify the genes that were dynamically differentially expressed, the RVM F-test was used.

2. ***Gene Ontology (GO):***

For the purpose of predicting the target gene of miRNAs, the TargetScan and miR Tar Base database was used. The ontology classification of genes, which is based on gene annotation and summary information that is available through DAVID (Database for Annotation, Visualization, and Integrated Discovery), was utilised in order to gain an understanding of the roles of predicted target genes. The projected target genes were categorised into their respective functional groups based on the molecular functions, biological processes, and specific pathways that they participate.

3. ***TF-miR-net:***

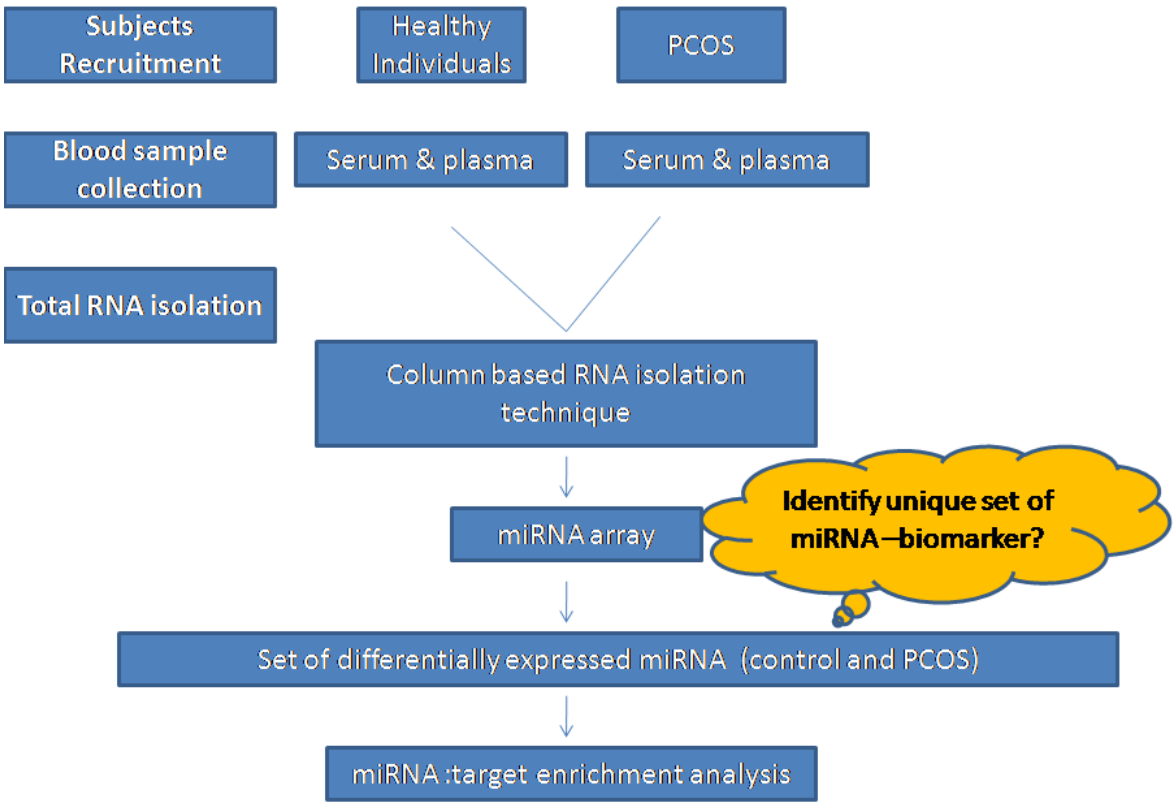
A mapping of the miRNA sequence onto the genome was performed in the Sanger database. In order to investigate the alignment of pre-miR sequences and potential transcription factor binding sequences, the

Jemboss software was utilised. The network that shows the interaction between transcription factors and miRNAs was built with the use of a genome browser database. Java, which is a computer language, was used to create an adjacency matrix that was organised according to the binding of pre-miRNAs and transcription factors. The core transcription factor of the network was the most critical centre and had the highest degree. By determining the degree of correlation that exists between transcription factors and miRNAs, the Pearson correlation analysis was applied in order to evaluate the degree to which transcription factors can regulate gene expression.

4. MicroRNA-gene-network:

The interaction between miRNAs and target genes were the foundation for the creation of the MicroRNA-Gene Network. Eight miRNAs were selected based on the pathways that were retrieved from KEGG and used as major nodes in the network. The number of nodes in the network that had a degree of 10 or higher was used to determine the relevance of the link between miRNAs and target genes within the context of the MicroRNA-Gene Network.

Expression of selected circulatory miR in serum:



Data was entered in Excel sheet. Statistical analysis was done by SPSS 23 software using

descriptive statistics for summarizing the frequency, percentage, mean and standard deviation of the data with 95% confidence interval and comparison of the different groups using independent t-test was calculated at 5% level of significance.

Significance of the proposed research and expected outcome :

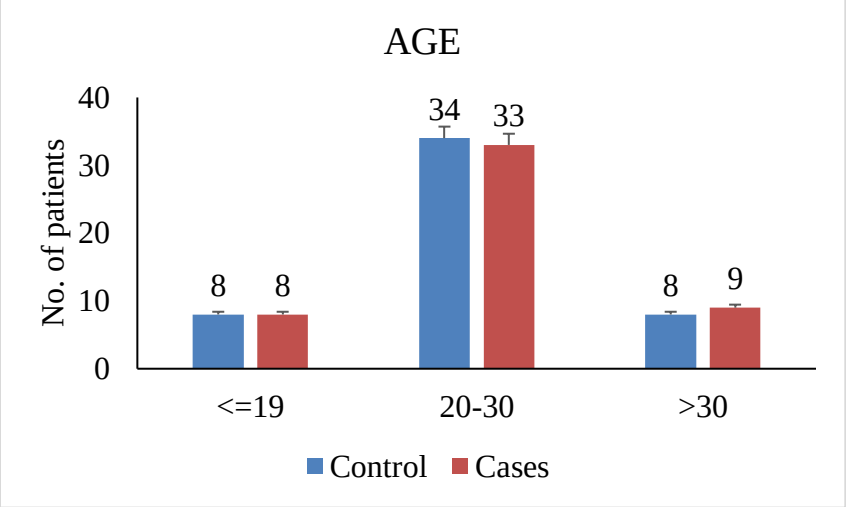
miRNAs could act as novel minimally invasive biomarker for diagnosis of PCOS. Although miRNAs are known to regulate multiple gene targets in different system they can also be regulated by other paracrine/autocrine factors. Understanding the mechanism that regulate the expression of the miRNAs in subjects with PCOS could identify new therapeutic targets in these women and help to achieve personalized medicine .This would further help to understand complex phenotypes of PCOS and advance the design of therapeutic approaches which would ameliorate major co morbidities like Type 2 Diabetes Mellitus,Metabolic syndrome, Cardio-Vascular Disease, endometrial cancer and so forth in later life. This could be used for genetic counselling in these patients.

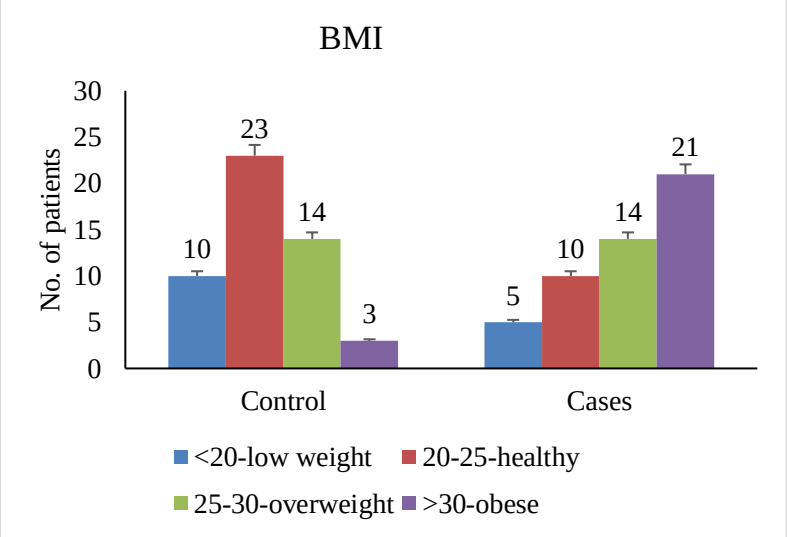
Results:-Statistical Analysis: Chi-Square Test: -

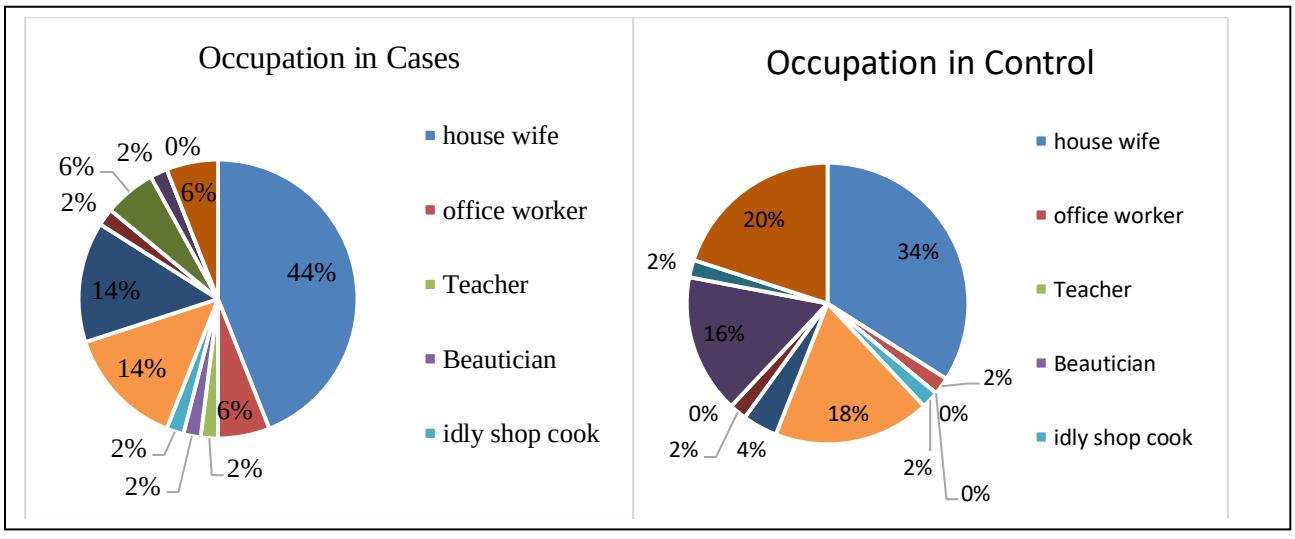
Comparison of socio-demographic variable between Control and Case Group.

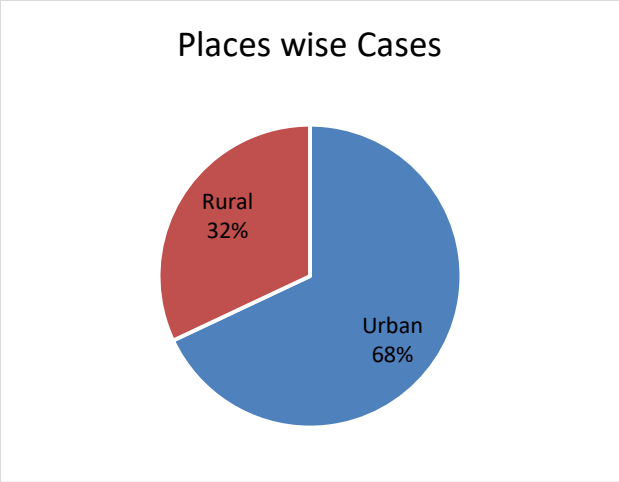
Age	**Case n (%)**	**Control n (%)**	**P value**
≤ 19	8 (16)	8 (16)	0.96
20-30	33(66)	34 (68)	
>30	9(18)	8 (16)	
BMI	**Case n (%)**	**Control n (%)**	**P value**
<20-low weight	5(10)	10 (20)	<0.01*
20-25-healthy	10(20)	23 (46)	
25-30-overweight	14(28)	14 (28)	
>30-obese	21(42)	3 (6)	
Occupation	**Case n (%)**	**Control n (%)**	**P value**
housewife	22(44)	17(34)	0.03*
office worker	3(6)	1(2)	
Teacher	1(2)	0(0)	
Beautician	1(2)	0(0)	
idly shop cook	1(2)	1(2)	
Student	7(14)	9(18)	
Factory worker	7(14)	2(4)	
shop worker	1(2)	1(2)	
Tailoring	3(6)	0(0)	
health care worker	1(2)	8(16)	
Agriculture worker	0(0)	1(2)	
Labourer	3(6)	10(20)	

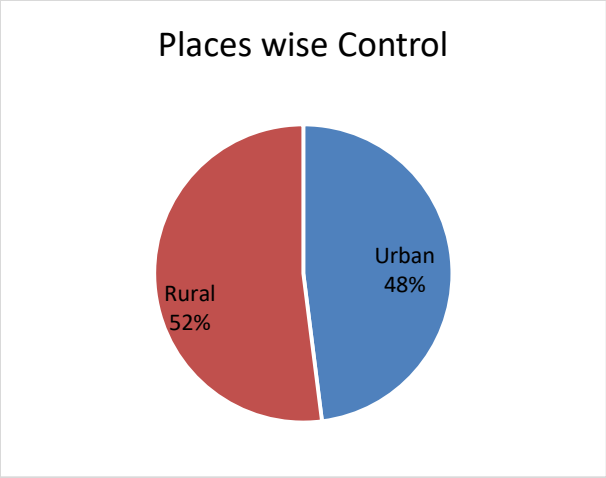
Place	Case n (%)	Control n (%)	P value
Urban	34(68)	24(48)	0.04*
Rural	16(32)	26(52)	







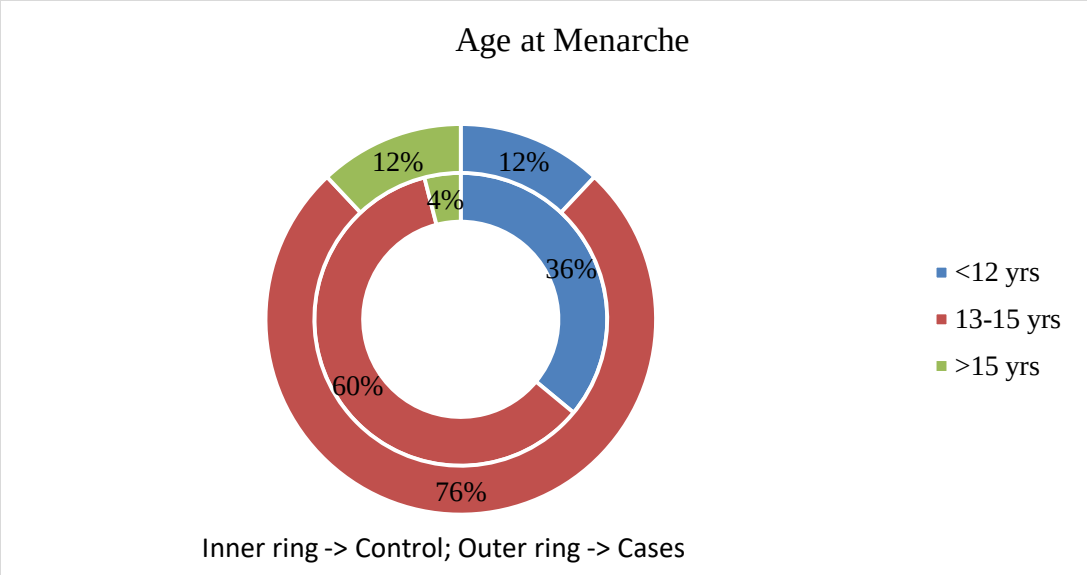


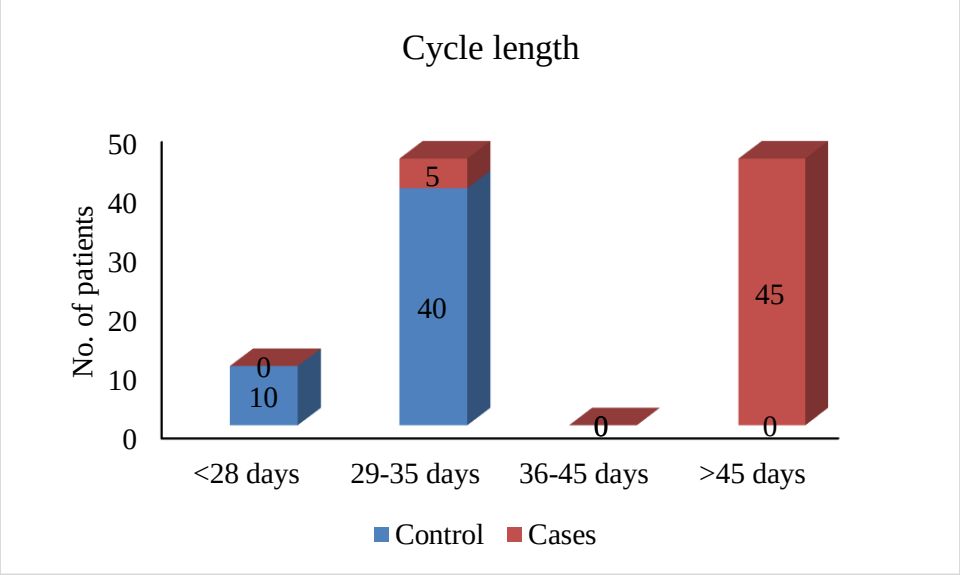


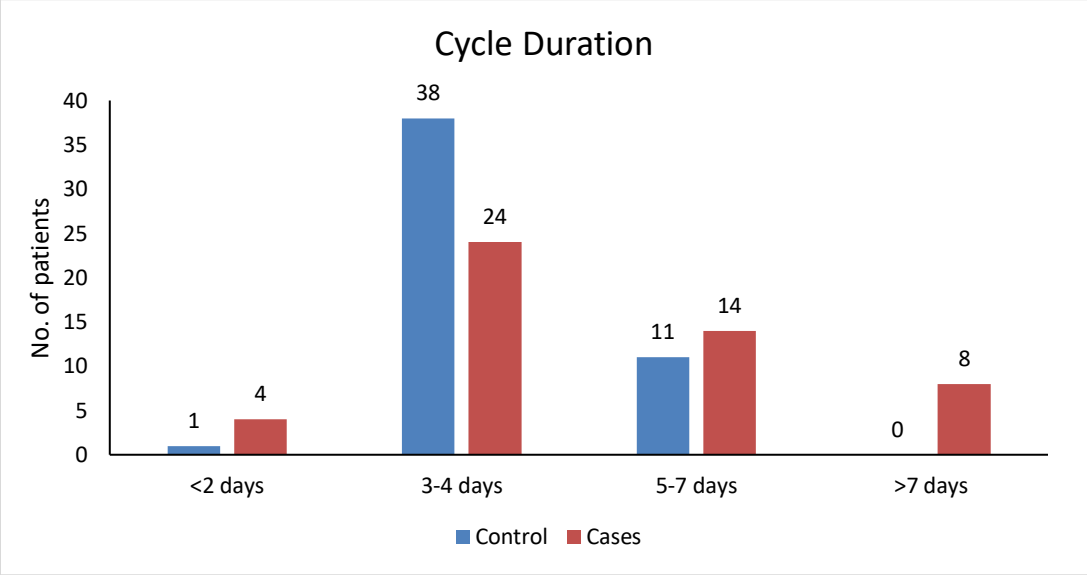
As the case and controls were age matched there was no statistical difference in age between cases and controls. The number of women with high BMI was higher in PCOS cases when compared to controls and the difference was statistically significant .With regard to occupation there were more housewives and office workers in PCOS cases compared to controls and the difference was statistically significant. PCOS cases were more from urban than rural area and this was again statistically significant.

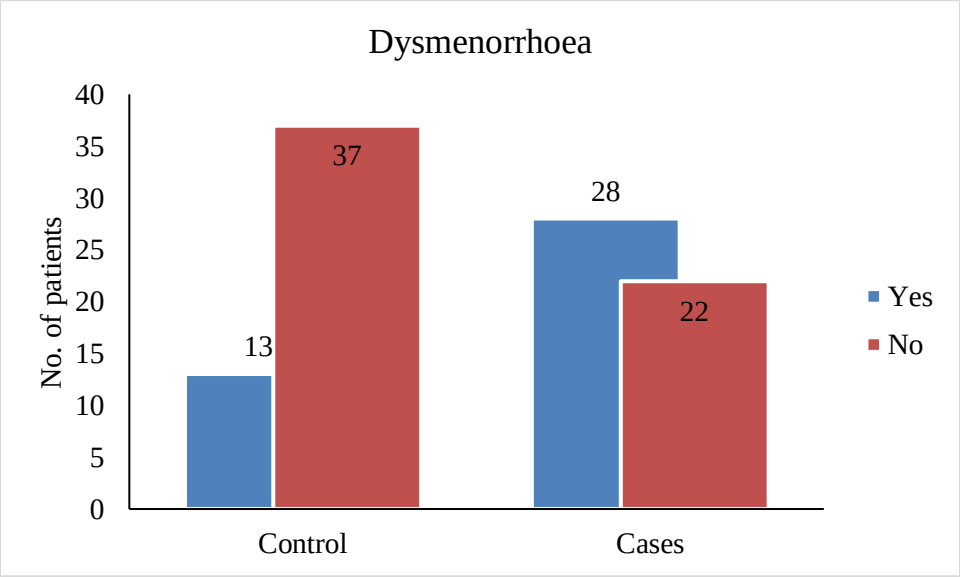
Comparison of menstrual related variable between Control and Case Group.

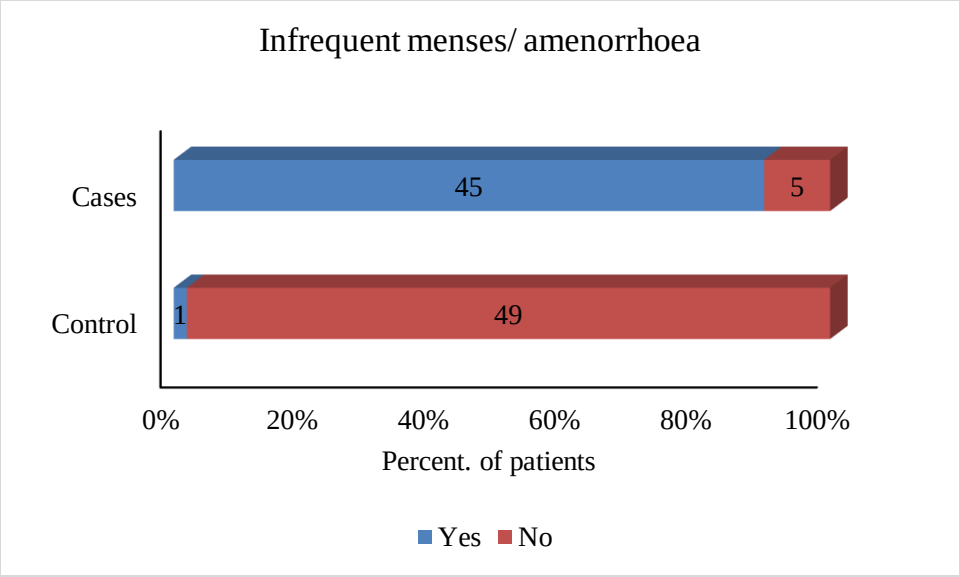
Age at Menarche	**Case n (%)**	**Control n (%)**	**P value**
<12 yrs	6(12)	18(36)	0.01*
13-15 yrs	38(76)	30(60)	
>15 yrs	6(12)	2(4)	
Cycle length	**Case n (%)**	**Control n (%)**	**P value**
<28 days	0(0)	10(20)	<0.01*
29-35 days	5(10)	40(80)	
36-45 days	0(0)	0(0)	
45 days	45(90)	0(0)	
Cycle duration	**Case n (%)**	**Control n (%)**	**P value**
< 2 days	4(8)	1(2)	0.004*
3-4 days	24(48)	38(76)	
5-7 days	14(28)	11(22)	
7 days	8(16)	0(0)	
Dysmennorrhoea	**Case n (%)**	**Control n (%)**	**P value**
Yes	28(56)	13(26)	0.002*
No	22(44)	37(74)	
Amenorrhoea	**Case n (%)**	**Control n (%)**	**P value**
Yes	45(90)	1(2)	<0.01*
No	5(10)	49(98)	


Age at Menarche
12%
12%
4%
36%
60%
76%
<12 yrs
13-15 yrs
>15 yrs
Inner ring -> Control; Outer ring -> Cases


Cycle length
No. of patients
50
40
30
20
10
0
0
10
5
40
0
45
0
<28 days
29-35 days
36-45 days
>45 days
Control
Cases


Cycle Duration
No. of patients
40
35
30
25
20
15
10
5
0
1
4
38
24
11
14
0
8
<2 days
3-4 days
5-7 days
>7 days
Control
Cases

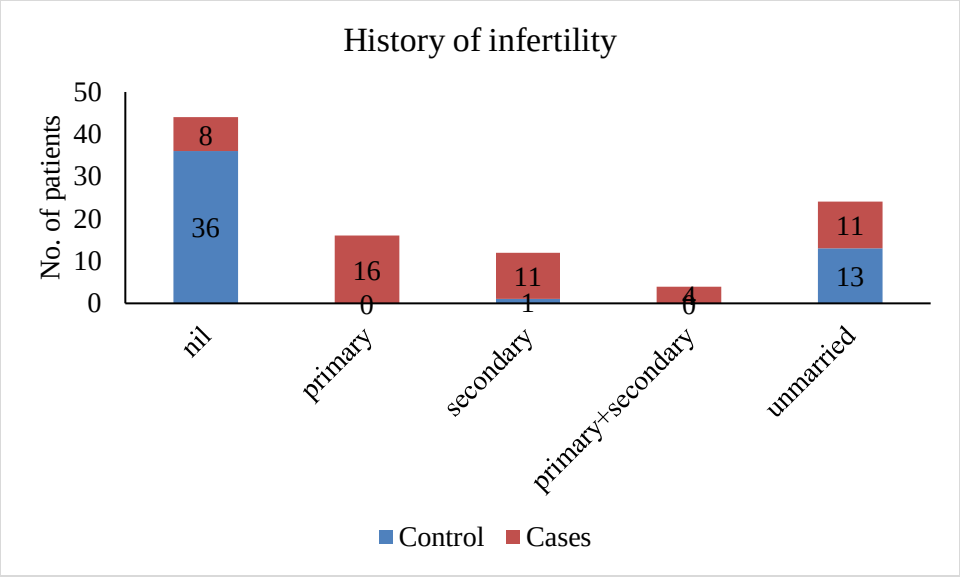

Dysmenorrhoea
No. of patients
40
35
30
25
20
15
10
5
0
13
37
28
22
Control
Cases
Yes
No

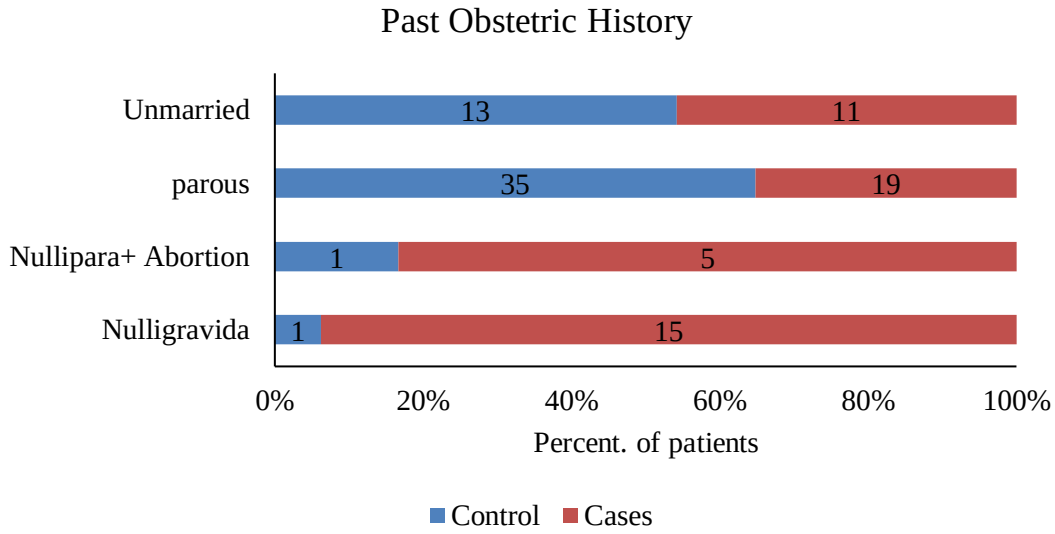


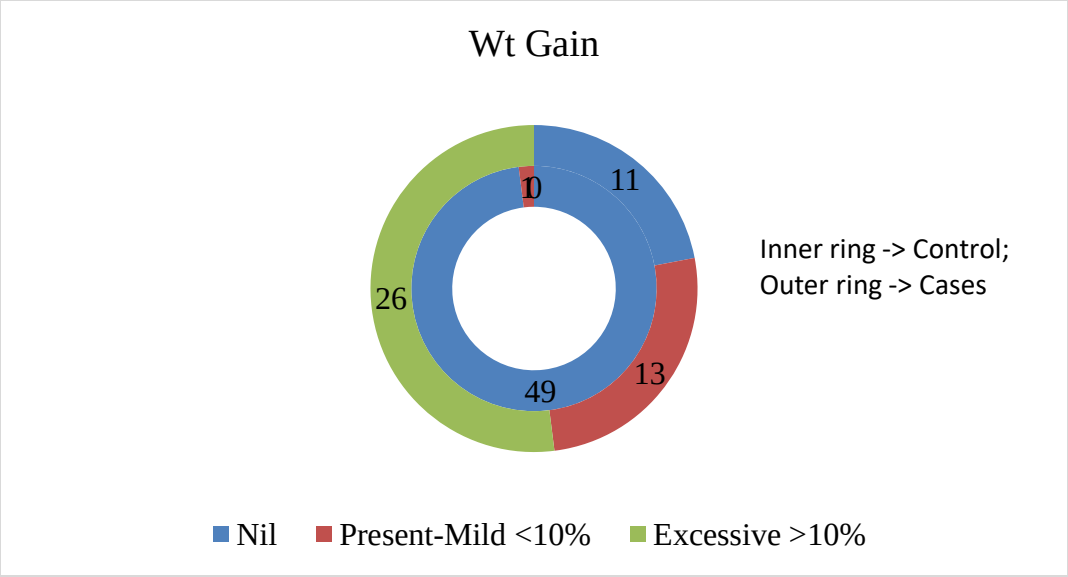
PCOS cases had a statistically significant slightly later age at menarche compared to controls. Cycle length was more than 45 days and the cycle duration was more in a statistically significant proportion of PCOS cases when compared to controls. A statistically significant number of PCOS cases had dysmenorrhoea and amenorrhoea when compared to controls.

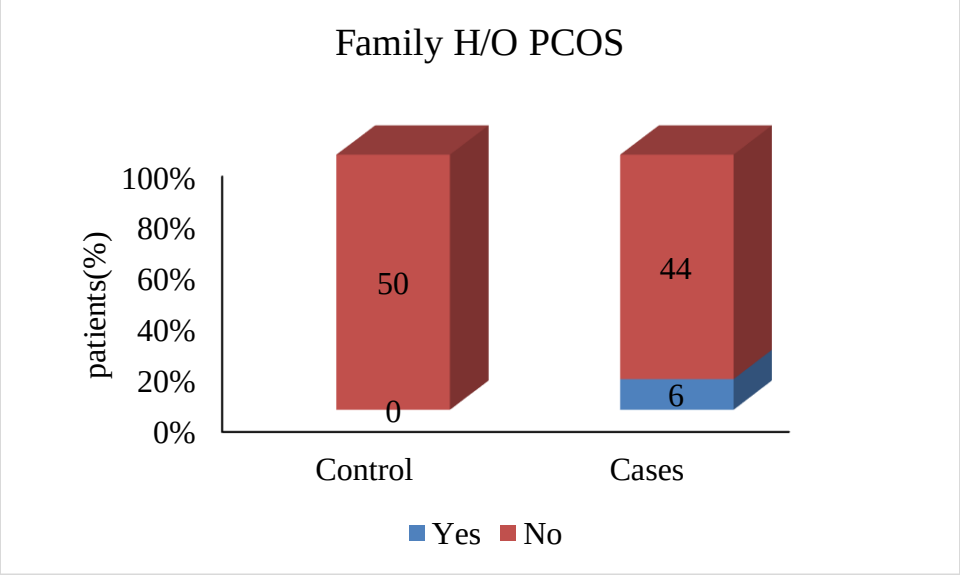
Comparison of past information variables between Control and Case Group.

History of Infertility	**Case n (%)**	**Control n (%)**	**P value**
Nil	8(16)	36(72)	<0.01*
Primary	16(32)	0(0)	
Secondary	11(22)	1(2)	
Primary+Secondary	4(8)	0(0)	
Unmarried	11(22)	13(26)	
Past Obsteric	**Case n (%)**	**Control n (%)**	**P value**
Nulligravida	15(30)	1(2)	<0.01*
Nullipara+Abortion	5(10)	1(2)	
Parous	19(38)	35(70)	
Unmarried	11(22)	13(26)	
Weight Gain	**Case n (%)**	**Control n (%)**	**P value**
Nil	11(22)	49(98)	<0.01*
Present-Mild <10%	13(26)	1(2)	
Excessive>10%	26(52)	0(0)	
Family PCOS	**Case n (%)**	**Control n (%)**	**P value**
Yes	6(12)	0(0)	0.012*
No	44(88)	50(100)	


History of infertility
No. of patients
50
40
30
20
10
0
8
36
16
0
11
1
4
0
11
13
nil
primary
secondary
primary+secondary
unmarried
Control
Cases


Past Obstetric History
Unmarried
parous
Nullipara+ Abortion
Nulligravida
13
11
35
19
1
5
1
15
0%
20%
40%
60%
80%
100%
Percent. of patients
Control
Cases

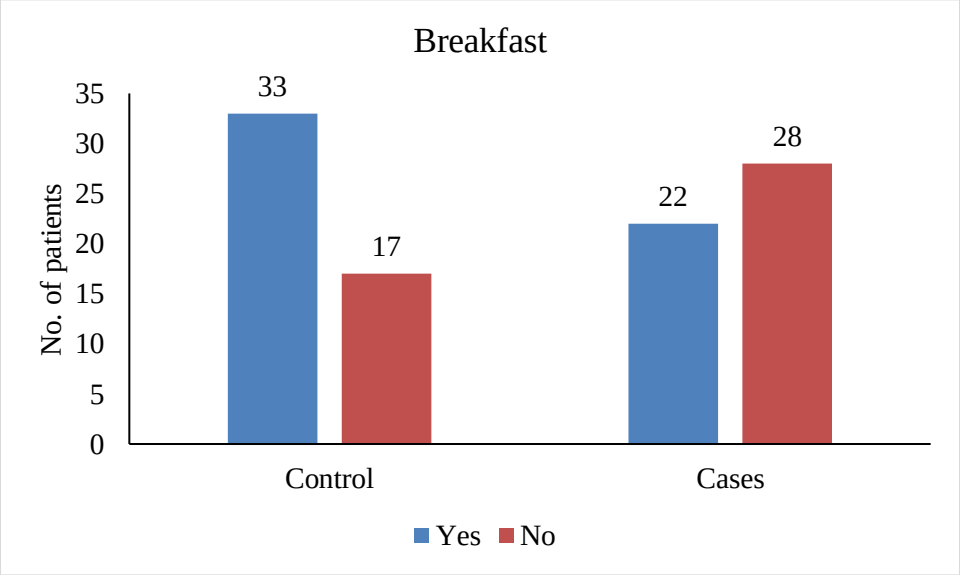


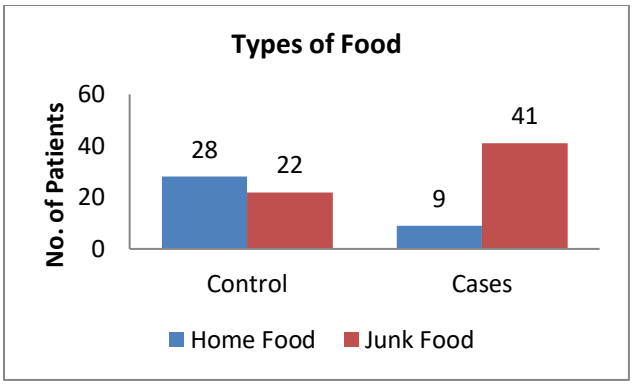


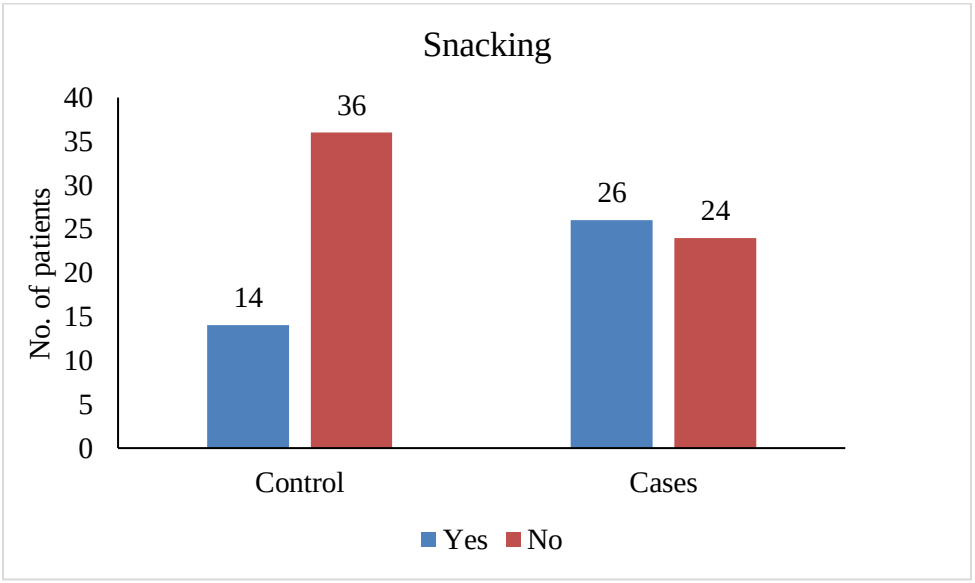
As expected there was statistically significant number of PCOS cases with infertility and a greater number of nulliparous women among PCOS women when compared with controls .There was a statistically significant increase in weight gain in the last one year in PCOS women when compared to controls and there was also a statistically significant increase in family history of PCOS in women with PCOS when compared to control.

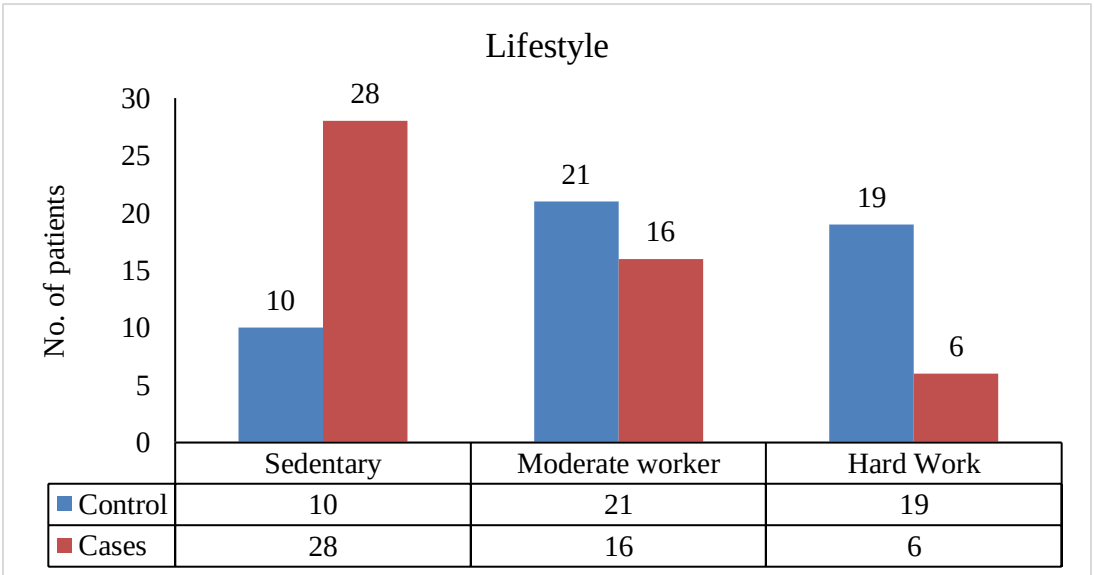
Comparison of Food Habit and Life Style variables between Control and Case Group.

Breakfast completion	**Case n (%)**	**Control n (%)**	**P value**
Yes	22(44)	33(66)	0.027*
No	28(56)	17(34)	
Type of Food	**Case n (%)**	**Control n (%)**	**P value**
Home Food	9(18)	28(56)	0.005*
Junk Food	41(82)	22(44)	
Snacking	**Case n (%)**	**Control n (%)**	**P value**
Yes	26(52)	14(28)	0.014*
No	24(48)	36(72)	
Life Style	**Case n (%)**	**Control n (%)**	**P value**
Sedentary	28(56)	10(20)	<0.01*
Moderate workers	16(32)	21(42)	
Hard Work	6(12)	19(38)	




Types of Food
No. of Patients
60
40
20
0
28
22
9
41
Control
Cases
Home Food
Junk Food

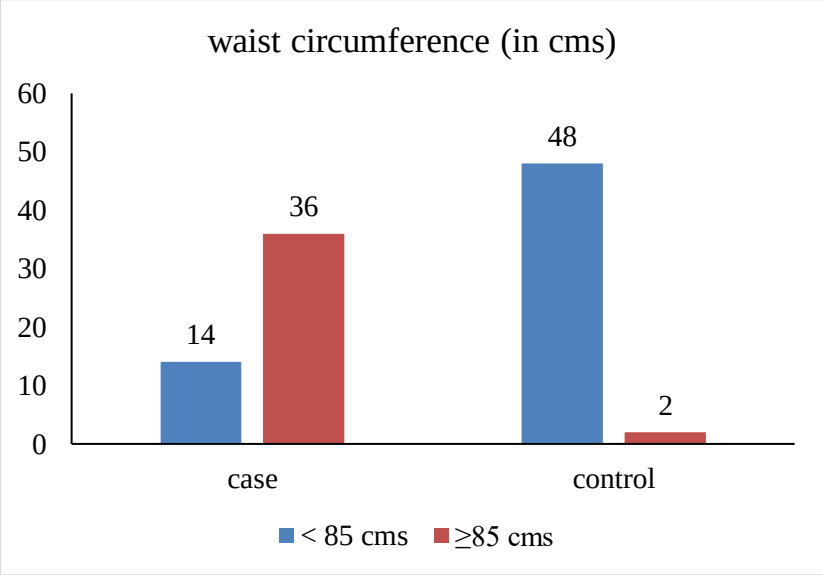

Snacking
No. of patients
40
35
30
25
20
15
10
5
0
14
36
26
24
Control
Cases
Yes
No

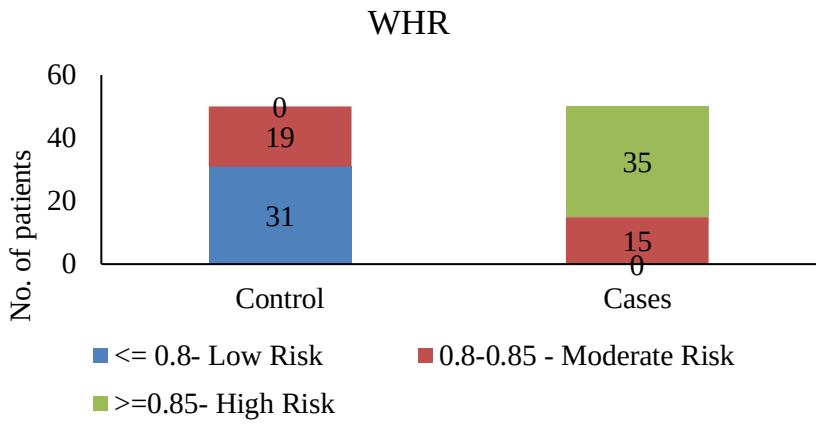

Lifestyle
No. of patients
30
25
20
15
10
5
0
10
28
21
16
19
6
Sedentary
Moderate worker
Hard Work
Control
10
21
19
Cases
28
16
6

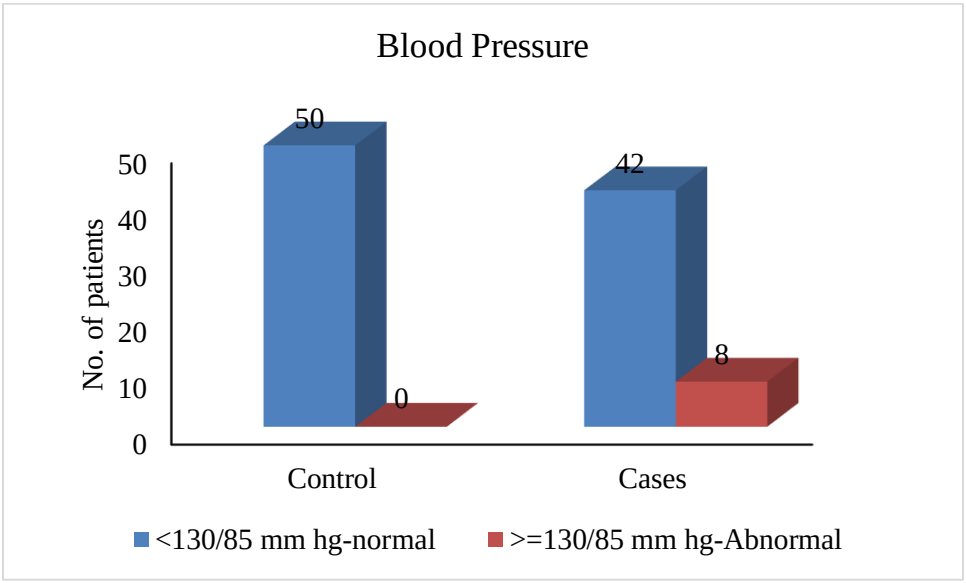
A statistically significant number of PCOS women skipped breakfast and more often had junk food when compared to control. Also a statistically significant number of PCOS women had a habit of snacking and led a more sedentary life when compared to the control women.

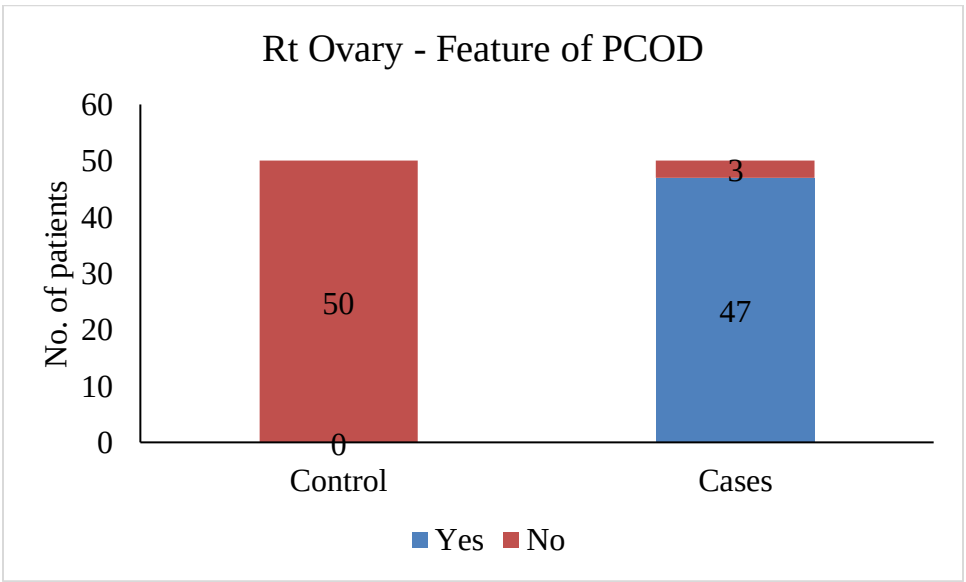
Comparison of Clinical variables between Control and Case Group.

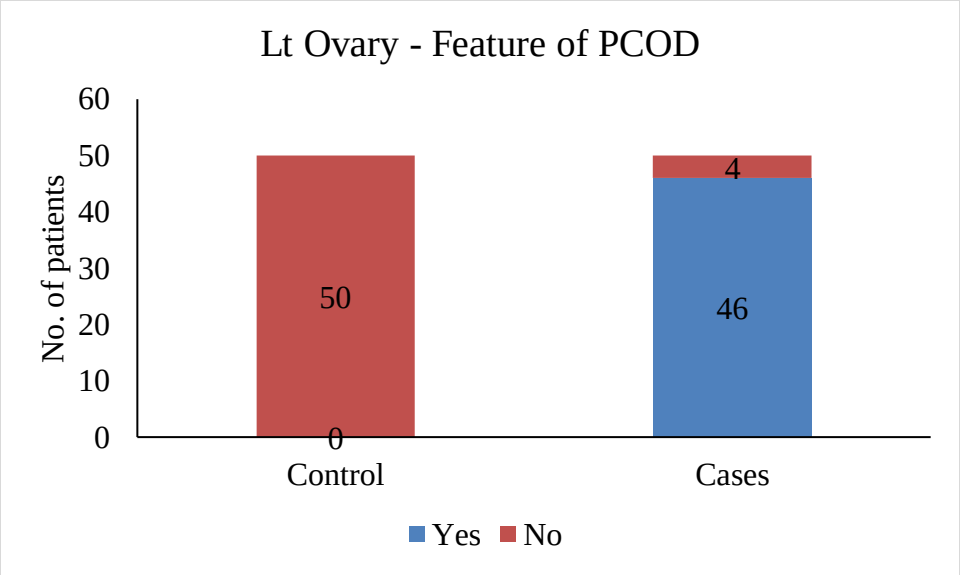
Waist circumference(cms)	**Case n (%)**	**Control n (%)**	**P value**
< 85 cms	14(28)	48(96)	<0.01*
≥85 cms	36(72)	2(4)	
Waist Hip Ratio	**Case n (%)**	**Control n (%)**	**P value**
≤0.8- Low Risk	0(0)	31(62)	<0.01*
0.8-0.85 - Moderate Risk	15(30)	19(38)	
≥0.85- High Risk	35(70)	0(0)	
Blood pressure	**Case n (%)**	**Control n (%)**	**P value**
<130/85mm hg normal	42(84)	50(100)	0.003*
≥ 130/85mm hg Abnormal	8(16)	0(0)	
USG-Rt ovary Features of PCOD	**Case n (%)**	**Control n (%)**	**P value**
Yes	47(94)	0(0)	<0.01*
No	3(6)	50(100)	
USG-Lt ovary Features of PCOD	**Case n (%)**	**Control n (%)**	**P value**
Yes	46(92)	0(0)	<0.01*
No	4(8)	50(100)	




WHR
60
40
20
0
No. of patients
0
19
31
35
15
0
Control
Cases
<= 0.8- Low Risk
0.8-0.85 - Moderate Risk
>=0.85- High Risk


Blood Pressure
50
40
30
20
10
0
No. of patients
50
0
42
8
Control
Cases
<130/85 mm hg-normal
>=130/85 mm hg-Abnormal


Rt Ovary - Feature of PCOD
60
50
40
30
20
10
0
No. of patients
50
0
3
47
Control
Cases
Yes
No



The waist circumference was more than 85 cm in a statistically significant number of PCOS women and the waist –hip ratio was in the high risk group in women with PCOS when compared to controls. There was a statistically significant increase in the number of PCOS women with Blood pressure above 130/85mm hg. There was a statistically significant increase in the number of PCOS women with ultrasound findings of PCOS in at least one ovary as it was one of the criteria for inclusion in the study.

Independent Sample t test:

	Case		**Control**		**p value**
	N	Mean ± SD	N	Mean ± SD	
Age of patient	50	24.84±5.46	50	24.94±5.31	0.9
No of years married	50	5.46±5.35	50	4.22±4.55	0.215
Height in cms	50	153.94±5.74	50	156.8±6.07	0.017*
Weight in kgs	50	66.69±15.18	50	58.12±10.55	0.002*
BMI	50	29.08±6.68	50	23.79±3.82	<0.01*
Hirsutism	50	8.2±3.02	50	4.1±0.84	<0.01*
Waist Circumference in cms	50	89.78±12.57	50	67.74±10.19	<0.01*
Hip circumference in cms	50	103.38±14.07	50	85.25±11.4	<0.01*
Serum Free Testosterone (in ng/ml)	50	0.38±0.15	50	0.27±0.06	<0.01*
Serum Sex Hormone Binding Globulin (in nmol/it)	50	31.53±26.05	50	36.64±10.34	0.201
FAI	49	5.25±2.51	50	2.63±0.63	<0.01*
Rt Ovary Vol	50	13.35±3.89	50	4.66±1.77	<0.01*
Lt Ovary Vol	50	12.47±2.96	50	4.31±1.63	<0.01*

*Significant at 0.05 level

There was a statistically significant increase in the number of women who had a Ferriman –Gallwey score of more than 8 in PCOS women when compared to control .The serum free testosterone, serum sex hormone binding globulin and the Free Androgen Index (FAI) was more in PCOS women when compared to controls and the difference was statistically significant. The ovary volumes were more than 10 cc as it was once again the criteria for inclusion in the study according to Rotterdam criteria.
Among 864 differentially expressed microRNAs (control vs PCOS), only 24 miRNAs (4 upregulated, and 20 downregulated) with p>0.05 was selected for enrichment analysis to identify gene targets using mirTar Base and Target Scan. In the enrichment analysis, 965 gene targets were obtained and it was filtered to 428 based on statistical significance, p>0.05. The number of target genes was further filtered based on the number of interactions with miRNAs to obtain significant miRNA for interaction network analysis. Targets showing 3 or more interactions were selected, from which 8 miRNAs, such as miR-382-5p, miR-370-5p, miR-107, miR-193b-3p, miR-15b-5p, miR-1-3p, miR-218-5p and miR-379-5p were used for functional network analysis. Dot plot and heat map from KEGG, Reactome and Wiki network was obtained from functional enrichment analysis which showed significantly over represented pathways (q<0.05) of the selected miRNAs using miR Tar Base and Target Scan. MiR-15b-5p (67 hits) and miR-1-3p (22hits) showed the highest number of consensus targets in KEGG network from Target Scan and miR Tar Base respectively as observed from Dotplot. MAPK signaling, oocyte meiosis, endocrine resistance, endometrial cancer, inositol phosphate metabolism and breast cancer were some of the enriched pathways associated with PCOS in KEGG network (Fig.1). The eight selected miRNAs used for miRNA-gene interaction network in KEGG and HPO revealed pathways related to Poly cystic ovaries, Type II diabetes mellitus, metabolic pathways, P13K and Akt signaling pathways were most associated with PCOS (Fig.2 and 3).

Fig.1: Overrepresented pathways (q < 0.05) of the selected miRNAs in KEGG

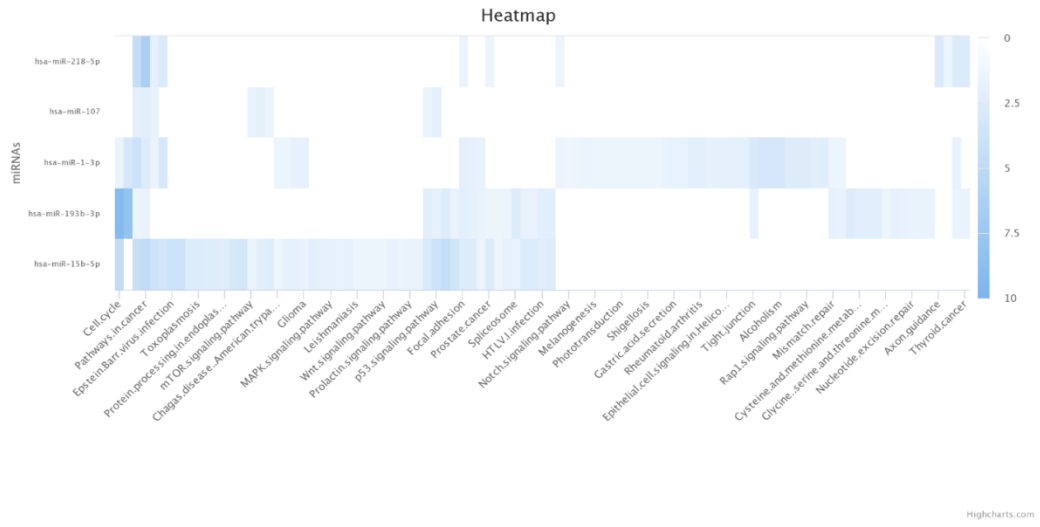
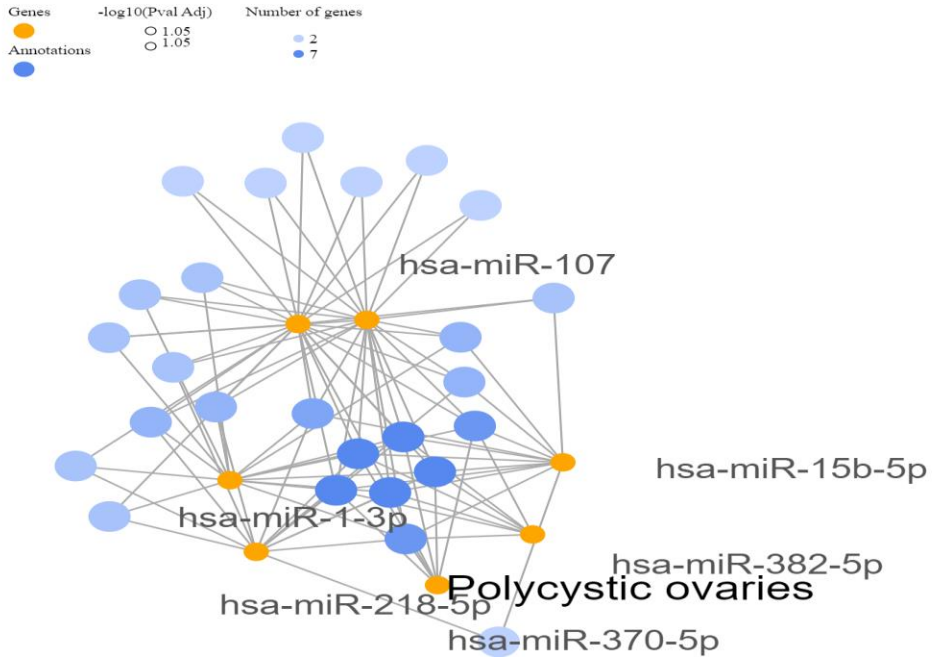


Fig.2: MiRNA-gene interaction network in HPO



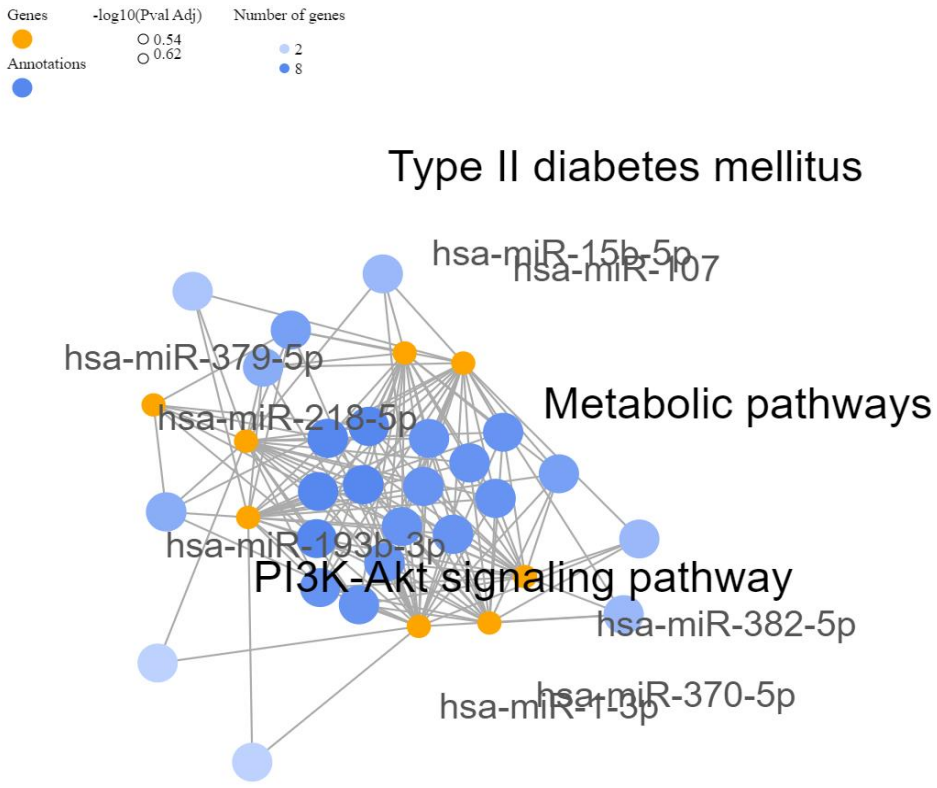


Fig.3: MiRNA-gene interaction network in KEGG

Discussion:

Reported studies on polycystic ovary syndrome (PCOS) have mostly focused on investigating the differential expression of a number of miRNAs[15]. Early research in this field found that patients with PCOS had elevated levels of several microRNAs, including miR-21, miR-103, miR-155, miR-16, miR-222, miR-106b, miR-19a, miR-146a, miR-30c, miR-186, miR-24 and miR-27b, which are known to play a role in the development and progression of PCOS[5, 12-13]. During in vitro maturation of mouse follicles, it has been shown that miR-27a, miR-let-7c, and miR-322 regulated the meiotic competence of oocytes. On the other hand, miRNA-21, miRNA-27b, miRNA-103, and miRNA-155 have been linked with disrupted hormone metabolism, irregular menstrual cycle, and insulin signalling associated with PCOS. The majority of these microRNAs that were dysregulated in PCOS target genes such as StAR, CYP19, CYP11A, and CYP17 are involved in the synthesis of steroid hormones[8]. Targeting CYP19 allowed miR-378 and miR-181 to reduce aromatase protein levels which in turn led to a drop in estradiol levels. Because of these cumulative effects, there is an increase in the amount of bioactive testosterone, while there is a decrease in the levels of sex hormone binding globulin molecule (SHBG). The influence of exosomes and their cargoes which may contain miRNAs that can regulate the process of inflammation, angiogenesis, and steroidogenesis all contribute to the progression of the disease. ExomiRs, miR-132, and miR-320 are associated with the initiation of steroidogenesis, and overweight/obese women with PCOS showed a decline in all three of these miRNA expression. PCOS women with prominent exosome levels showed a strong association with follicular counts and insulin resistance markers in the ovary[6]. ExomiRs miR-146a-5p and miR-126-3p have been found to be elevated in PCOS, while miR-20b-5p, miR-106a-5p and miR-18a-3p expression showed a significant decline. Genes and pathways associated with axon guidance, MAPK signalling, endocytosis, and circadian rhythms are targeted by these differentially expressed exomiRs[6]. The Indian research network has helped to identify miRNAs that are differently expressed in PCOS, just like the foreign researcher. According to Dhaded et al[16], miR-21 and miR-146 are considerably elevated in PCOS and miR-92a, miR-19b, and miR-200a are considerably downregulated. Sathyapalan et al[17] reported an increase in MiR-93 and miR-223 in PCOS when compared to controls of similar age. Additionally, their research aimed to establish these miRs as fresh biomarkers for detecting PCOS. However, neither insulin resistance nor the rise of miR-93 and miR-223 were related to each other or hyperandrogenaemia, which are symptoms of PCOS, reducing their effectiveness as therapeutic agents serving as diagnostic markers efficiently. The baseline data was from 58 patients, with 29 of them having PCOS and 29 of them served as normal controls.All patients were obese, their ages and BMIs were comparable, and they differed significantly with regard to the levels of insulin, insulin resistance (HOMA), testosterone, free FAI, and AMH(Anti-Mullerian Hormone) in their systems. One hundred and seventy-six different miRNAs were found, and out of those, only 15 showed significant

differences between normal women and PCOS patients. MiR-486-5p, miR-24-3p, miR-19b-3p, miR-22-3p, miR-19a-3p, miR-339-5p, miR-185-5p, miR-101-3p, and miR-let-7i-5p were found to be statistically significant ($p<0.02$)[18]..

Summary and conclusion:

In our study women with PCOS had a significant association with increased BMI, history of weight gain, unhealthy food habits and sedentary life style compared to controls. They also had a significantly increased waist circumference, Waist hip ratio and Hypertension along with increased incidence of metabolic syndrome.

8 miRNAs, such as miR-382-5p, miR-370-5p, miR-107, miR-193b-3p, miR-15b-5p, miR-1-3p, miR-218-5p and miR-379-5p were found to be differentially expressed in PCOS women with $P>0.05$ with three or more target interactions. Hence these differentially expressed microRNAs could be used as novel biomarkers to identify women with tendency to develop PCOS before they develop the disease and by counselling them and by bringing about life style modifications like healthy food habits, yoga and exercise they can prevent the onset of PCOS with its multiple reproductive and metabolic complications and thus enable them to have good quality of life. Understanding the mechanism that regulate the expression of the miRNAs in subjects with PCOS could identify new therapeutic targets in these women and help to achieve personalized medicine

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